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ANALYSIS OF FATTY ACID PEROXIDE TYPES IN
MIXTURES OF OLEATE, LINOLEATE, AND LINOLENATE
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Analysis of fatty acid peroxide types in mixtures

of oleate, linoleate, and linolenate esters

by

Seyed Hassan Fatemi

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INTRODUCTION

Peroxide values often are used to measure the extent of oxidation of lipids, but peroxide values give a composite measure when more than one fatty acid is present so that one does not know how much each of the fatty acids in the mixture has oxidized. Each fatty acid can give rise to different sorts of flavors through the scission of the hydroperoxides; so to understand the role that the different fatty acids play in the development of off-flavors in fats and oils, it is vital to know the relative rates of oxidation of the fatty acids in mixtures.

In a previous attempt in this laboratory (65), a simple and reliable method was worked out for the quantitative analysis of the hydroperoxide types in autoxidized methyl oleate and linoleate mixtures. The method involved the reduction of the hydroperoxide, silylation of the resulting hydroxyester, and analysis by gas chromatography. In the present study, this procedure was extended to mixtures of methyl oleate, linoleate, and linolenate and also to randomized and natural oils, to study the possible effect of triglyceride structure on the oxidation rates of fatty acids.

REVIEW OF LITERATURE

The oxidation of unsaturated substances has been the subject of numerous investigations. For a long time, it was considered that the first products formed in the autoxidation of fatty acids and their esters were cyclic peroxides. In 1928, Stephens (59) reported the isolation of a peroxide of cyclohexene which he obtained by treating cyclohexene with oxygen in daylight. He assumed, on the basis of theories of oxidation accepted at that time, that the product was saturated. During the early forties, Farmer (14) and Farmer and Sundralingam (16) formulated the currently accepted mechanism of autoxidation. They showed that Stephen's product was a hydroperoxide and that a double bond was present. According to Farmer, the oxidation of practically all unconjugated olefin compounds proceeds by a chain reaction involving addition of a molecule of oxygen to the carbon atom adjacent to the double bond to form a hydroperoxide with an intact double bond. Later, Farmer (15) and others (6) modified their theory to admit that autoxidation chains are initiated by a reaction of the oxygen at the double bonds, but the chains are propagated by reactions at the alpha-methylenic positions. Hilditch (29) suggested a mechanism in which oxygen adds directly to the double bond, forming a transitory cyclic peroxide, which then may rearrange to hydroperoxide. More recently, Khan (37) suggested that oxygen attacks the unsaturated molecules directly, forming a six-membered cyclic complex. This complex constitutes the transition state through which the hydroperoxide could be formed.

The geometrical and positional isomers obtained during autoxidation

have been extensively studied by many researchers. Farmer and Sutton (17) isolated methyl oleate peroxide by continuous molecular distillation. This somewhat impure peroxide consisted mainly of mono-hydroperoxide, but contained a little di-hydroperoxide together with some peroxide transformation product. Knight and Swern (40), by using infrared spectrophotometry, found most of the peroxides produced during the autoxidation of methyl oleate were _trans_ peroxides. This indicated that _cis-trans_ isomerization occurred during the initial stage of autoxidation. Later, this result was confirmed by Khan et al. (39). They suggested that conversion of _cis_ to _trans_ double bonds and peroxide formation occurred in the same molecule and hence no inversion of the double bond occurred in unoxidized oleate. Ross et al. (55) reported shift of double bond in the autoxidation of methyl oleate. They also found four possible isomers as predicted by Farmer's theory. However, Hall and Roberts (26) by using nuclear magnetic resonance (NMR) and infrared (IR) found no evidence of 11-hydroperoxide of oleate. Recently, Piretti et al. (51) reported obtaining four isomers in mixtures of hydroperoxides from methyl oleate autoxidized in a current of air at temperatures of 20, 40, and 80 C. These isomers were somewhat different from those predicted by Farmer's theory. They assumed that the formation of these isomers can be due to allyl isomerization of peroxide radical. The structural investigation of autoxidation products of methyl oleate has been carried out by Frankel et al. (19) by gas chromatography-mass spectrometry (GC-MS) of trimethylsilyl (TMS) ether derivatives. They found consistently higher concentration of the 8- and 11-hydroperoxides than of the 9- and 10-hydroperoxides. According to Frankel (18), the most accepted mechanism for oleate autoxidation involves hydrogen

abstraction at the carbon-8 and -11 positions. By this mechanism, equal amounts of 8-, 9-, 10-, and 11- isomers are expected. Since allylic hydroperoxides are known to undergo allylic rearrangement (8), Frankel et al. suggested that this isomerization may provide an adequate explanation for the uneven distribution of isomers found in their study (19).

Many studies have been carried out to find the position of the hydroperoxide group in methyl linoleate hydroperoxide. Bergstrom (3), after hydrogenating autoxidized methyl linoleate, was able to isolate 9- and 13-mono-hydroxystearates but not 11-mono-hydroxystearates. His results, therefore, left it an open question whether any 11-hydroperoxide or other unconjugated hydroperoxide is formed to any appreciable extent during the autoxidation of methyl linoleate. Bolland and Gee (6) made quite extensive studies of the kinetics of autoxidation of ethyl linoleate. After making calculations based on the bond and resonance energies that are involved, they suggested that there would be a tendency against the formation of any appreciable amount of the unconjugated 11-hydroperoxide. Bolland and Koch (7) postulated the formation of a resonating linoleate free radical by the removal of a hydrogen atom from the methylenic carbon between the two double bonds. In addition to its unconjugated form, this radical could assume either of two conjugated dienoic forms which could be stabilized by the formation of hydroperoxide groups at the 9 or 13 carbon positions. Cobern et al. (12) suggested that the hydroperoxide groups are located at each of the carbon atoms which originally formed part of a double bond. The absence of 11-hydroperoxide has been also reported by some other workers (5, 26, 58), but Khan et al. (38) and Lundberg et al. (44) found some 11-hydroperoxide in the autoxidation of

methyl linoleate. Bank et al. (1) reported equal amounts of 9- and 13-hydroperoxides. Chan and Levett (10) by high performance liquid chromatography (HPLC) and adsorption chromatography could separate and identify four major isomers as 9- and 13-positional isomers having the trans-trans and cis-trans configurations. Recently, the separation by silver nitrate thin-layer chromatography (TLC) of isomeric mono-hydroperoxides produced from autoxidized methyl linoleate has been reported (63). Neff et al. (49) by using HPLC identified different secondary products of autoxidation of methyl linoleate.

The enzymatic oxidation of linolenic acid has been studied by Graveland (23). He found that lipoxygenase in wheat flour oxidized linolenic acid only at carbon 9, and that no oxidation took place at carbon 16, 12, and 13. Unlike linoleate autoxidation, which yields equal proportions of 9- and 13-positional isomers, the relative proportions of the four positional isomers obtained from linolenate autoxidation are not equal, but favor the 9 and 16 isomers by a ratio of 3 to 1 (11). This could be due to either the preferential attack of oxygen at positions 9 and 16 or to the higher rate of decomposition of 12- and 13-hydroperoxides.

Special conditions of autoxidation have been studied by some investigators. Swern et al. (61) in the autoxidation of methyl oleate oxidized at 35 to 120 C found some nonhydroperoxides, believed to be cyclic peroxides. Lundberg and Chipault (43) reported that at all levels of oxidation of methyl linoleate up to 300 meg/kg of peroxide and at all temperatures between 40 and 100 C, a constant fraction of the total peroxides is present as conjugated dienes. Cannon et al. (9) and Khan et al. (39) observed that during autoxidation of methyl linoleate conju-

gated cis-trans hydroperoxides are formed predominately at low temperatures
and conjugated trans-trans peroxides at room temperature. The trans-trans
molecules arise partly, at least, by thermal rearrangement of already
formed cis-trans peroxide (58). Equal amounts of 9- and 13-hydroperoxides
have been found in all samples of methyl linoleate oxidized at different
temperatures and peroxide levels (13, 18, 62). Khan et al. (38) found
some evidence for the formation of 11-hydroperoxide when linoleate was
oxidized in the presence of chlorophyll. The oxidation of linolenate has
been shown to be similar to linoleate in forming a cis-trans, conjugated
monomeric mono-hydroperoxide as a major initial product of autoxidation at
low temperatures (52).

The reduction of the hydroperoxide group to the corresponding unsaturat-
ed secondary alcohol is a fundamental step in the characterization of fat
hydroperoxides and has been investigated by many workers (31, 40).
Stannous chloride was used to determine peroxide quantitatively by Hargrave
and Morris (28). They found yields ranging from 42-100%. The iodometric
reduction was first used by Lea (42). It involves heating the oil or
fat with glacial acetic acid and chloroform in the presence of potassium
iodide solution and titrating the liberated iodine with thiosulfate.
Wheeler's technique (64), which is used by the American Oil Chemists'
Society, uses a saturated solution of potassium iodide and is performed
at room temperature in the presence of air. Barthel and Grosch (2), in
comparing the different methods of peroxide value determination, found 100%
recovery for methyl linoleate hydroperoxides by the Wheeler method.
Raghuveer (53) found essentially complete reduction of hydroperoxide
with potassium iodide, but only 60 to 80% recovery using sodium borohydride

or stannous chloride.

. The kinetics of olefin oxidation have been studied by Bolland (5),
Mayo et al. (46, 47), and Betts (4). Russel (56) has derived a relative
rate equation for co-oxidation of two hydrocarbons.

$$\frac{d(R_1H)}{d(R_2H)} = \frac{d(R_1OOH)}{d(R_2OOH)} = \frac{r_1(R_1H)/(R_2H) + 1}{r_2(R_2H)/(R_1H) + 1}$$

In this equation, RH represents an olefin, and r_1 and r_2 are ratios of the
rate constants of the chain propagation reactions. Mayo and Lewis (45)
used an integrated form of the equation to determine the ratios graphically.
A simplified and more practical linear method for determining the reactivi-
ty ratio was later published by Ross (54).

The early works on the oxidation rates of different unsaturated fatty
acids and esters were mostly based on peroxide value determinations and
oxygen absorption which confuse initiation and propagation rates. Stirton
et al. (60) studied the oxidation of oleate-linoleate and linoleate-lino-
lenate mixtures by measuring the oxygen absorption. They found that
linoleate absorbed oxygen 10.5 times faster than oleate, and the oxidation
rate of linolenate was twice that of linoleate. Holman and Elmer (32)
suggested that the increase by one in the number of double bonds in a
fatty acid or its ester increases the rate of oxidation by at least a
factor of two. The abstraction of hydrogen from the methylene group α to
the double bond needs less energy in a 1, 4-diene system than monoene be-
cause the hydrogen is activated by two double bonds, so the rate of
autoxidation of linoleic acid is faster than that of oleic acid. Howard
and Ingold (33, 34) using the rotating sector apparatus found the ratio
of absolute rate constants for oleate:linoleate:linolenate of 1:23:44.

Yanishlieva and Popv (66) by measuring the concentration of conjugated diene and peroxide values in mixtures of oxidized methyl oleate and linoleate, found no oleate hydroperoxides in mixtures containing less than 50% methyl oleate.

Recently, Frankel and his co-workers have studied the cooxidation of some unsaturated fatty acid esters extensively by GC-MS analysis of TMS derivatives of the reduced hydroperoxides. In equal mixtures of oleate and linoleate, about 80% of the peroxides formed at three peroxide levels originated from linoleate, and with a 9:1 oleate-linoleate mixture, 50% of the hydroperoxides formed came from linoleate (20). They found that the 9-hydroxy ester came from both oleate and linoleate hydroperoxides, but the 13-hydroxy ester came only from linoleate hydroperoxides. With a 9:1 linoleate-linolenate mixture, 32% of the hydroperoxides originated from linolenate, and with the equal oleate-linoleate-linolenate mixtures, 52% of hydroperoxides were derived from linolenate and 39% from linoleate (21). More recently, Frankel and Neff (22) in the study of soybean oil esters oxidized at low peroxide levels, found an unusual isomeric distribution of hydroperoxides. The unexpected high concentration of the 12-hydroperoxide isomer was in marked contrast to the isomeric composition of oxidized pure linolenate. They suggested that these different isomeric hydroperoxides at low levels of oxidation may contribute through their decomposition to the unique flavor deterioration of soybean oil.

In previous attempts in this laboratory, different methods were used to study the cooxidation effects of fatty ester mixtures. Raghuveer (53) developed a procedure to isolate and analyze peroxides from mixtures of fatty acid esters or natural oils. This method involved the acetylation

of the methyl hydroxy esters with acetic anhydride, and the resulting methyl acetyl esters were separated from unoxidized esters by urea fractionation. The oxidized esters were measured by charring with sulfuric acid on a thin layer silica gel plate and densitometry of the charred spots. Johnson (36) simplified this procedure by substituting butyryl for acetyl side chains. After debutyrylation of the methyl esters, he could separate the methyl hydroxyesters on a silica gel plate impregnated with silver nitrate, and by charring the plate and densitometry, the oxidized fatty esters were determined. Since Johnson's results could not be completely repeated, Wong (65) took an entirely new approach to solve this problem. He prepared a TMS-derivative of the hydroxyesters of oleate and linoleate and by gas chromatography determined the amount of each oxidized fatty acid esters. He did not find a constant relation between the oxidation rate of oleate and linoleate mixtures, but in general, linoleate was oxidized about 10 times faster than oleate.

In this study, the silylation procedure was extended to mixtures of oleate-linoleate-linolenate and also to randomized and natural oils.

MATERIALS AND METHODS

Materials

Methyl oleate was prepared from olive oil. First, mixed methyl esters were obtained by refluxing 400 g of olive oil with 1,000 ml of methanol for 4 h in the presence of 0.5% sodium methoxide as catalyst. Mixed methyl esters were extracted with hexane, and the hexane layer was washed free of base and dried over sodium sulfate. The hexane was removed under reduced pressure. Methyl oleate was isolated from olive oil methyl esters by urea fractionation following chiefly the procedure of Raghuveer (53) as shown in the flow diagram. Urea adduct was dissolved in 2N hydrochloric acid and the esters were extracted with hexane. The hexane extract was washed free of acid, dried with sodium sulfate, and the solvent was evaporated. The methyl esters obtained in this fraction were analyzed by gas chromatography (GLC) and contained 96.6% oleate and 3.4% palmitate.

Methyl linoleate was isolated from safflower oil methyl esters by the urea fractionation procedure of Wong (65). The product was 99% pure.

Methyl linolenate was prepared from linseed oil. Mixed methyl esters were obtained by the same procedure discussed for olive oil, but transesterification was carried out for 6 h in the presence of 2% sulfuric acid as a catalyst. The linseed oil methyl esters were subjected to urea fractionation (50). This yielded a product containing 75.7% linolenate, 19.8% linoleate, and 4.5% oleate as determined by GLC. Another urea fractionation was performed on these methyl esters, and the product was 82.4% linolenate and 17.6% linoleate by GLC. Methyl linolenate of high purity also was obtained from Nu-Chek-PREP, Inc., Elysian, Minn.

Mixed olive methyl esters
3,400 ml methanol
1360 g urea
heated to boiling and cooled
to room temperature (r.t.)

urea adduct
recrystallization from 1.5 liters
methanol

filtrate
boiled down until
cloudy
cooled to r.t.

urea adduct
recrystallization

filtrate

urea adduct

filtrate
(discard)

boiled down until cloudy
cooled to r.t.

urea adduct
recrystallization

filtrate

urea adduct

filtrate
(discard)

boiled down until cloudy
cooled to r.t.

urea adduct
(discard)

filtrate

urea adduct

filtrate
(discard)

boiled down until cloudy
cooled to r.t.

urea adduct
(discard)

filtrate
boiled down until cloudy

urea adduct

filtrate
(discard)

All methyl ester samples were distilled at 0.5 mm pressure through a

44-cm Widmer column. Other impurities which interfered with subsequent

GLC of the silylated oxidation products were removed by passing the

samples through an alumina column (35). The methyl ester mixtures were prepared using pure methyl esters and methyl esters of natural oils. The ratio of fatty acids in each mixtures was checked by GLC.

Corn oil and soybean oil were randomized using 0.5% sodium methoxide. The reaction mixture was stirred with a magnetic stirrer for 5 h; the pressure was kept below 1 mm and the temperature was maintained at 60 C. After randomization, the mixture was washed with 5% acetic acid, 5% sodium bicarbonate solution, and finally with distilled water. The randomized oil was dried over sodium sulfate. Natural and randomized oils were purified only by passing through an alumina column.

Oxidative Conditions

All methyl ester mixtures and oils were oxidized in 5 g lots in 125-ml erlenmeyer flasks without stirring at 28 C, except those specified otherwise.

Samples were withdrawn periodically to determine the peroxide value (PV) by the Hamm et al. method (27). When samples reached peroxide values of approximately 5, 10, 20, and 40, samples were withdrawn and the peroxides were reduced to alcohols by the iodometric method of the American Oil Chemists' Society (48). The methyl esters were extracted three times with 100-ml portions of chloroform, washed once with distilled water and twice with 5% sodium bicarbonate solution. After drying the chloroform extract over sodium sulfate, the solvent was removed under reduced pressure. After reduction by iodide, the natural and randomized oils were transesterified in methanol using 0.5% sodium methoxide as a catalyst. The ratio of fatty acids was determined by GLC.

Thin-Layer Chromatography

Plates of silica gel G (0.75 mm thick) and silica gel G impregnated with 10% silver nitrate (0.5 mm thick), were used for preparative separations. They were air dried and activated at 100 C for 1 h. The silver nitrate plates were stored in a dark drying chamber.

To separate the methyl hydroxyesters from unoxidized methyl esters, 100 mg of the methyl ester mixtures were applied to a silica gel plate by a sample streaker (Applied Science Lab. Inc.). Methyl ricinoleate was applied to the sides of the plate as guide spots. The plate was developed in petroleum ether and ether (70:30; V/V), and after spraying the plate with 2% 2', 7'-dichlorofluorescein in 95% ethanol, the methyl hydroxyesters band was visualized under ultraviolet light. The band was scraped off the plate and extracted with 25 ml of ether, using the methyl ricinoleate guide spots to indicate the area to be extracted. The solvent was evaporated under a stream of nitrogen.

The methyl hydroxyesters mixtures were applied in a silver nitrate plate and the spot was kept as small as possible to enhance resolution. The plate was developed in petroleum ether and ether (40:60; V/V). The resolved spots were located by spraying the plate with 2', 7'-dichlorofluorescein and viewing under ultraviolet light. The resolved methyl hydroxyesters were extracted from the dichlorofluorescein-treated plate by the procedure of Hill et al. (30). For a more sensitive detection of spots when recovery of the esters was unnecessary, the plates were sprayed with 10% phosphomolybdic acid in 95% ethanol and charred at 110 C.

Trimethylsilyl (TMS) Ether Derivatives

Methyl hydroxyesters were dissolved in a mixture of 2 ml of hexane and 1 ml of pyridine, and 0.2 ml hexamethyldisilazane and 0.1 ml tri-methylchlorosilane were added with rapid mixing for 30 seconds. The esters were allowed to react for 4 h. After this, 5 ml of distilled water was added and the esters were extracted three times with 5 ml of hexane. The combined extracts were dried over sodium sulfate and the solvent was evaporated under a stream of nitrogen. For quantitative analysis, methyl heptadecanoate was used as the internal standard.

Gas-Liquid Chromatography

The GLC analysis was carried out on an F & M model or Beckmann GC% gas chromatograph equipped with a flame ionization detector. The temperature of the column (6 feet, 1/8 inch o.d.) was 180 C and the flow rates of nitrogen, hydrogen and air were 50, 50, and 300 cc/min respectively. The packing for methyl esters analysis was 15% EGSSX on 100/120 mesh Chromo-sorb P (Applied Science Lab. Inc.). For TMS-derivatives, 10% OV225 cyanopropyl silicone on 100/120 mesh Chromosorb W(HP) (Piece Chemical Company, Rockford, Illinois) was used.

Infrared Spectroscopy

IR spectra of TMS-linolenate were obtained with a Beckman IR12 infrared spectrophotometer. The analysis was performed in a sodium chloride ultra-micro liquid cell.

Mass Spectrometry

Mass spectra of TMS-linolenate before and after hydrogenation were obtained with a Finnigan Model 4000 gas chromatograph-mass spectrometer which was programmed from 200 to 280 C at 8 degrees/min.

Hydrogenation

Hydrogenation was carried out by bubbling the hydrogen through a reaction mixture containing 500 mg of linolenate hydroxy esters, 15 ml of methanol, and 100 mg of palladium on charcoal as a catalyst. The pressure was maintained at five pounds and the reaction was completed in 1 h. The catalyst was filtered off, the mixture was diluted with distilled water, and the product was then extracted with ether.

RESULTS AND DISCUSSION

Since Johnson's (36) separation of the methyl hydroxyesters obtained from oleate, linoleate, and linolenate hydroperoxides was not completely reproducible, the silylation procedure developed by Wong (65) was extended to the analysis of three fatty acids mixtures. The silylation method has been shown to give useful derivatives of hydroxyesters for convenient gas chromatography as well as mass spectrometry. The following scheme was used for isolation and quantitation of hydroperoxide types.

$$CH_3-(CH_2)_7-CH=CH-(CH_2)_7-COOCH_3$$

autoxidation

$$CH_3-(CH_2)_6-\underline{CH-CH-CH-CH}-(CH_2)_6-COOCH_3$$
$$\overline{O}OH$$

reduction $\qquad$ KI

$$CH_3-(CH_2)_6-\underline{CH-CH-CH-CH}-(CH_2)_6-COOCH_3$$
$$\overline{O}H$$

(1) TLC
(2) $AgNO_3$ TLC
(3) Silylation

$$CH_3-(CH_2)_6-\underline{CH-CH-CH-CH}-(CH_2)_6-COOCH_3$$
$$\overline{O}Si(CH_3)_3$$

GLC

Properties of TMS-Linolenate

The properties of TMS derivatives of oleate and linoleate had been thoroughly investigated by Wong (65). So, a similar study was made of the characteristics of TMS-linolenate.

Pure methyl linolenate was oxidized, reduced to methyl hydroxyesters, and fractionated by TLC. The methyl hydroxyesters were silylated. The GLC analysis of the silylated products gave four partially-separated peaks as shown in Figure 1. The IR spectrum indicated the presence of a TMS $Si(CH_3)_3$ group (845,1250 cm^{-1}), a methyl ester (1180,1745 cm^{-1}), cis-trans conjugated diene (950 cm^{-1}), and a trans-trans conjugated diene (988 cm^{-1}).

A mass spectrum of the TMS-linolenate showed fragments at mass 183, 223, 311, and 351 as reported by Frankel et al. (21), and correspond to the fragments in the following formula.

$$R = \left[-(CH_2)_7 - COOCH_3 \right]$$

A mass spectrum of hydrogenated TMS-linolenate confirmed the presence of four positional isomers: 9, 12, 13, and 16. The results are given in Table 1.

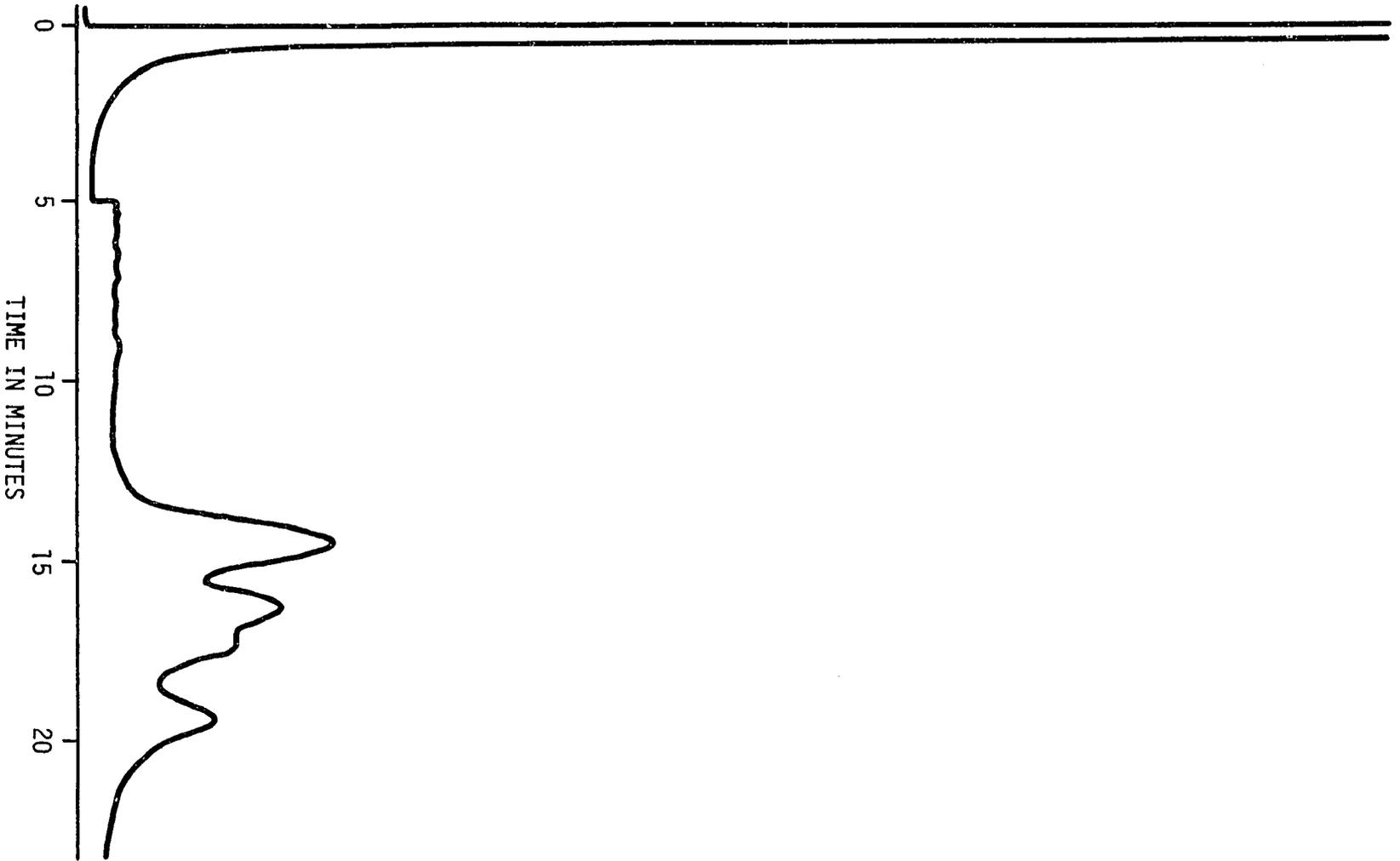Figure 1. Typical gas chromatogram of TMS-linolenate

RECORDER RESPONSE

TIME IN MINUTES

19

Table 1. Mass spectral data of hydrogenated TMS-linolenate

Characteristic fragments m/e (relative abundance)	Identification
229 (100), 259 (96.7), 371 (2.2)	9-OH
187 (100), 301 (36.1), 371 (2.1)	12-OH
173 (100), 315 (30.6)	12-OH
131 (100), 357 (36.7), 371 (1.1)	16-OH

Effectiveness of the Methods

To isolate hydroperoxides, they had to be first reduced to hydroxy-esters. Various reducing agents were tried and of these, potassium iodide gave the most satisfactory results. To test the efficiency of the reduction procedure, a sample of methyl esters after reduction with potassium iodide was applied to a silica gel plate. The plate was developed in petroleum ether and ether (70:30 V/V), and sprayed with an acidified solution of ammonium thiocyanate and ferrous sulfate (25). By this test, any unreduced hydroperoxides could be detected, but there was no evidence of the presence of hydroperoxides.

Preliminary trials showed that GLC would not separate TMS-linolenate from TMS-linoleate. Silver ion chromatography of the TMS-compounds before GLC analysis also failed to give a satisfactory separation. Satisfactory resolution was obtained by applying about 1 mg of the methyl hydroxyesters mixture as a tiny spot to a 0.5 mm-thick silica gel plate impregnated with 10% silver nitrate. The plate was developed in ether and petroleum ether (60:40 V/V). Applying larger quantities of the sample resulted in loss of resolution. The R_f values were dependent on the original

concentrations of each component. Figure 2 shows a typical silver ion thin-layer chromatogram for oleate, linoleate and linolenate.

Wong's experiments on the GLC analysis of the TMS derivatives showed a 63% yield for the oleate and 40% for the linoleate derivatives compared with a methyl heptadecanoate standard. To check his results, we analyzed oxidized olive oil methyl esters using these correction factors. However, the results did not fall on the curve obtained by Wong for the co-oxidation of oleate and linoleate. So, the correction factors for oleate and linoleate were redetermined along with the factor for linolenate.

Prolonged storage of the TMS-derivatives enhanced the chance of degradation (65). The TMS samples were stored at 5 C until GLC analysis. All GLC analyses were carried out within a few hours after reaction.

Determination of the Response Factors

Pure methyl oleate, linoleate, and linolenate were oxidized to peroxide values of approximately 5, 10, 20, and 40. After reduction and fractionation by TLC, methyl hydroxyesters were converted to TMS-derivatives. The GLC yield was determined using methyl heptadecanoate as the internal standard. Apparent yields (GLC response compared with peroxide value and sample size) for methyl oleate, linoleate and linolenate were found to be 62%, 44%, and 17% respectively (Figure 3). These correction factors were used during all subsequent analyses.

Oxidation of Binary Mixtures of Fatty Acids

A series of oleate-linoleate and linoleate-linolenate mixtures were oxidized to peroxide value of approximately 40, and the peroxides formed

Figure 2. Thin-layer chromatogram of methyl hydroxyesters on silver
nitrate-silica gel G. Solvent system: ether and petroleum
ether (60:40 V/V)

A. Hydroxymonoene

B. Hydroxydiene

C. Hydroxytriene
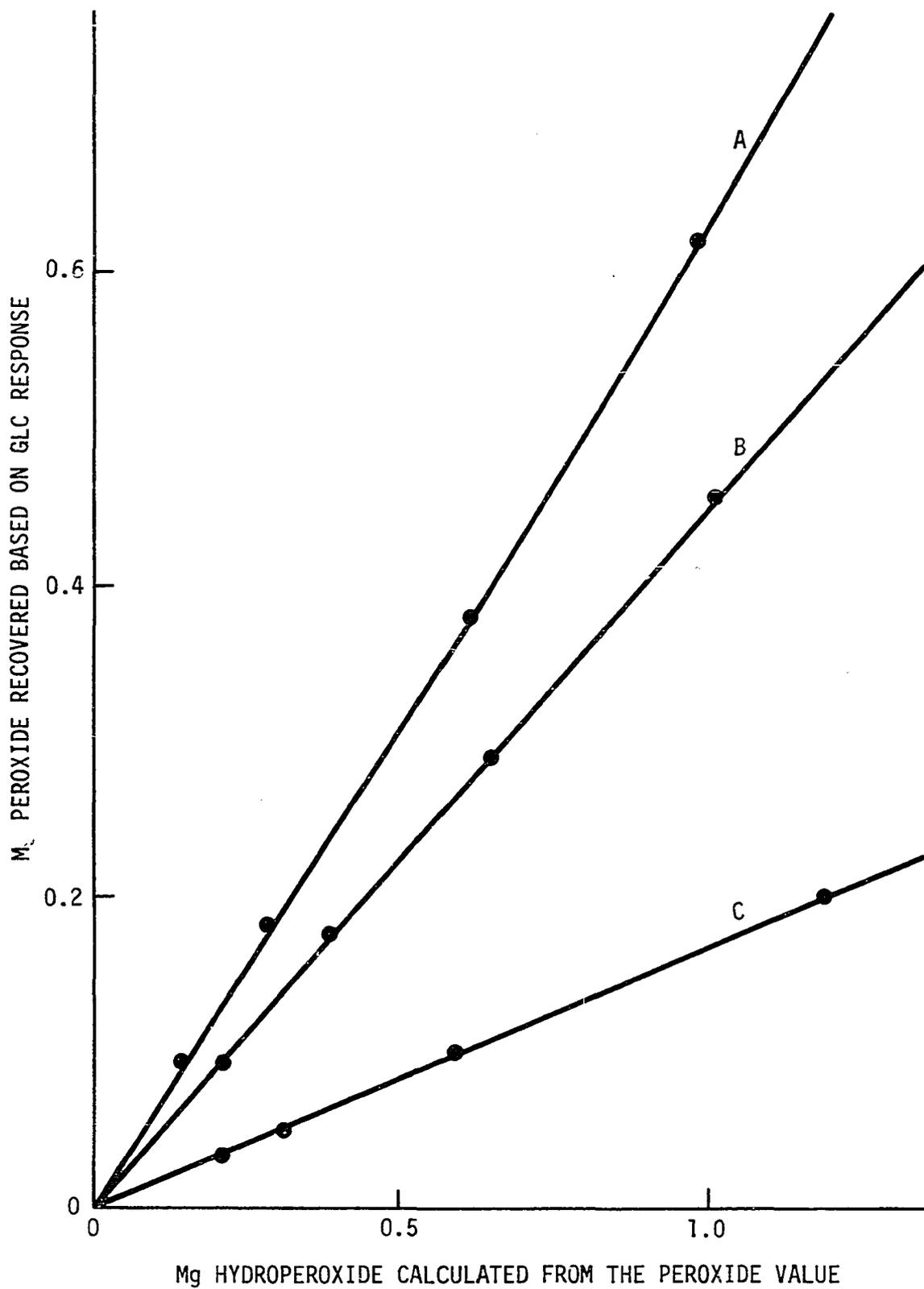
Solvent Front

A   B   C

Origin

Figure 3. Calculation of the recovery of TMS-derivatives from oxidized
esters

    A. Methyl oleate

    B. Methyl linoleate

    C. Methyl linolenate

were analyzed quantitatively.

In the mixtures containing at least 40% oleate, the oxidation of oleate-linoleate mixtures (Table 2) gave almost a consistent relation between the percentage of the hydroperoxide product of a fatty acid and its concentration in the mixture. In these mixtures, linoleate was oxidized about ten times faster than oleate. However, in mixtures containing 38% and 19% oleate, linoleate was oxidized six and four times faster than oleate, respectively. The results are plotted in Figure 4. Wong (65) noticed higher oxidation rate of oleate at lower concentration of this fatty acid. He attributed this effect, at least partly, to the incomplete resolution of oleate and linoleate peaks at higher proportions of linoleate. In these experiments, complete resolution was achieved (Figure 5), but the presence of small amounts of an impurity with the same retention time as oleate was noticed during the gas chromatography. This small peak was observed even in the mixtures of linoleate-linolenate, when there was no oleate, and it could account for the deviation in the amount of oxidized oleate when the concentration of this fatty acid in the mixture was small.

Very consistent rates of oxidation were found in linoleate-linolenate mixtures (Table 3). According to these results, linolenate should oxidize about two times faster than linoleate. The results are plotted in Figure 6.

In a binary mixture such as linoleate and linolenate, there are four propagation reactions:

Table 2. GLC analysis of the formation of peroxide types in autoxidized oleate-linoleate mixtures

Composition methyl ester mixture		P.V.	Recovery %	Peroxide %		Corrected peroxide ratio[a]
18:1	18:2			Monoene	Diene	18:2/18:1
87.1	12.9	25.3	92.1	38.15	61.84	10.94
77.31	22.69	33.3	91.2	25.64	74.36	9.9
68.51	31.49	34.4	86.04	19.57	80.43	8.95
57.98	42.02	41.5	93.6	13.04	86.98	9.2
38.65	61.35	47.4	88.5	8.42	91.57	6.04
19.73	80.27	57.4	103.7	5.55	94.45	4.2

[a]Ratio = % Diene peroxide x % Methyl oleate/ % Monoene peroxide x % Methyl linoleate.

Figure 4. Oxidation of linoleate at different ratios of oleate-linoleate
         concentration

% LINOLENATE HYDROPEROXIDE IN THE TOTAL HYDROPEROXIDES
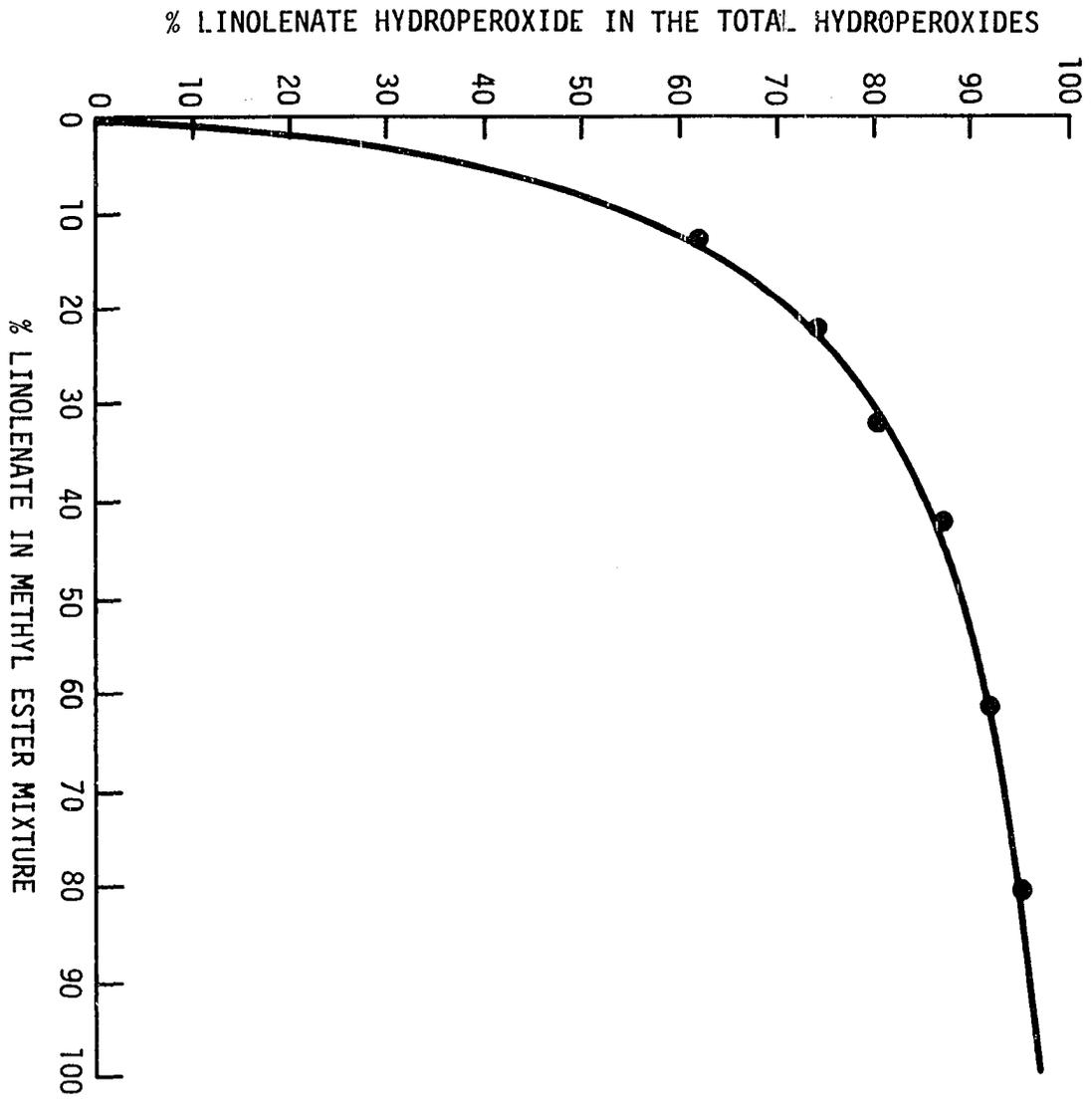
% LINOLENATE IN METHYL ESTER MIXTURE

Figure 5. Typical gas chromatogram of TMS-ester mixture

      A. TMS-oleate

      B, C, D. TMS-linoleate

RECORDER RESPONSE

TIME IN MINUTES

0

5

10 — A

B

15 — C

D

20
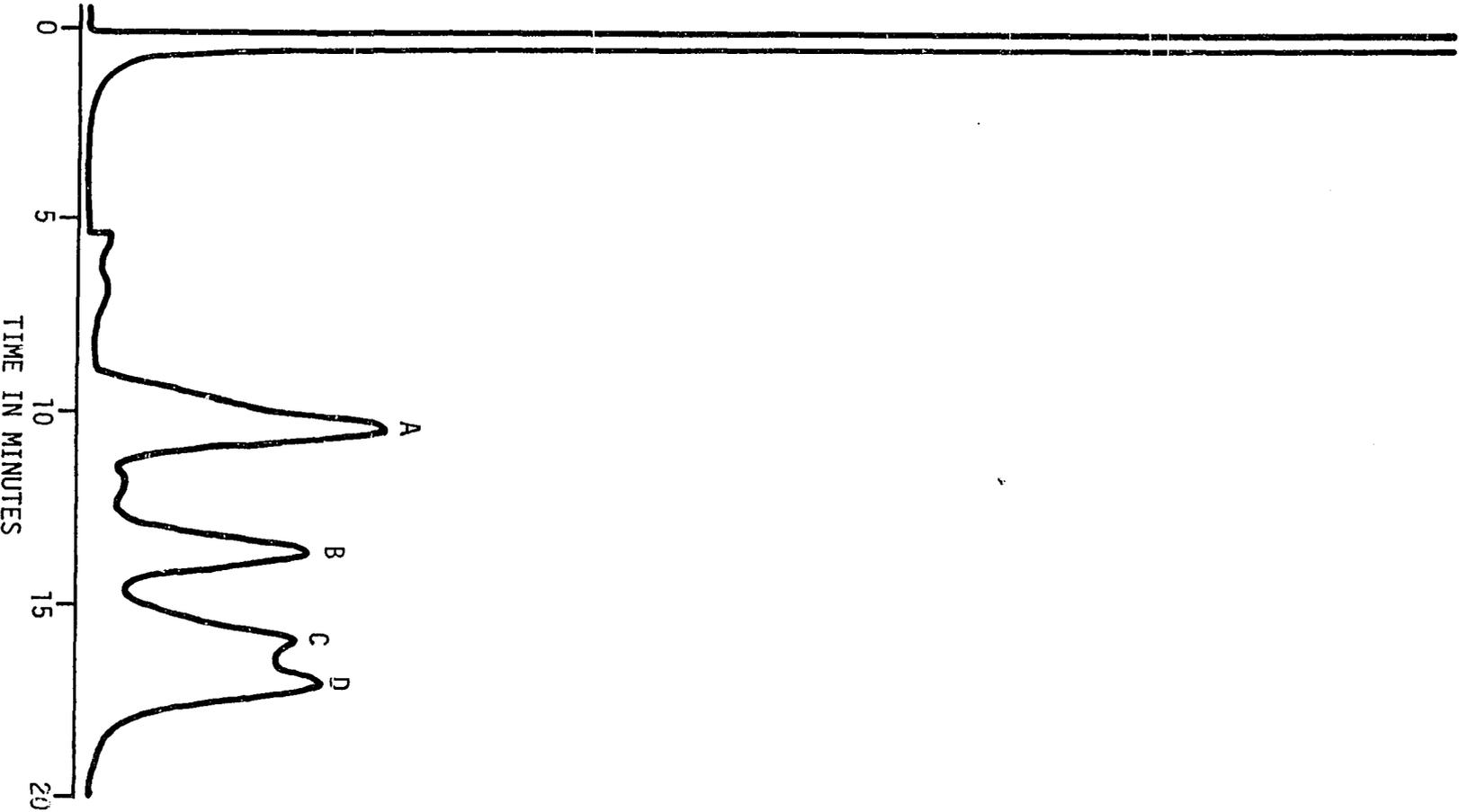
Table 3. GLC analysis of the formation of peroxide types in autoxidized linoleate-linolenate mixtures

Composition methyl ester mixture		P.V.	Recovery %	Peroxide %		Corrected peroxide ratio[a]
18:2	18:3			Diene	Triene	18:3/18:2
89.66	10.34	55.8	89.3	80.14	19.86	2.14
69.77	30.23	41.9	90.4	51.73	48.27	2.15
49.16	50.84	34.5	89.4	30.98	69.02	2.15
17.97	82.03	44.2	102.3	9.52	90.48	2.08

[a]Calculated as in Table 2.

Figure 6. Oxidation of linolenate at different ratios of methyl linoleate
and linolenate

% LINOLENATE HYDROPEROXIDE IN THE TOTAL HYDROPEROXIDES

% LINOLEATE IN METHYL ESTER MIXTURE

34

$$R_1OO. + R_1H \qquad R_1OOH + R_1. \qquad K_P^{11}$$

$$R_1OO. + R_2H \qquad R_1OOH + R_2. \qquad K_P^{12}$$

$$R_2OO. + R_2H \qquad R_2OOH + R_2. \qquad K_P^{22}$$

$$R_2OO. + R_1H \qquad R_2OOH + R_1. \qquad K_P^{21}$$
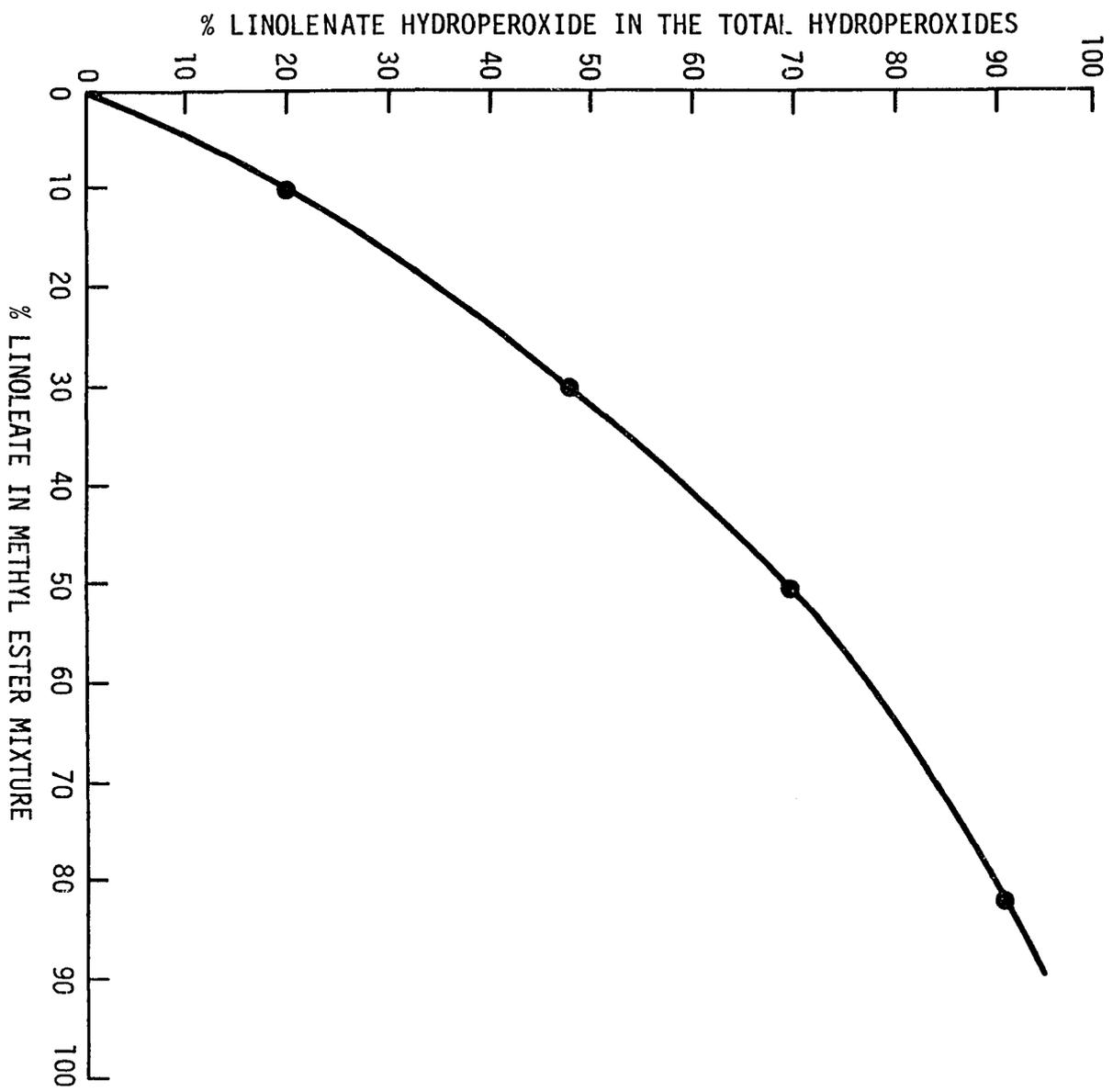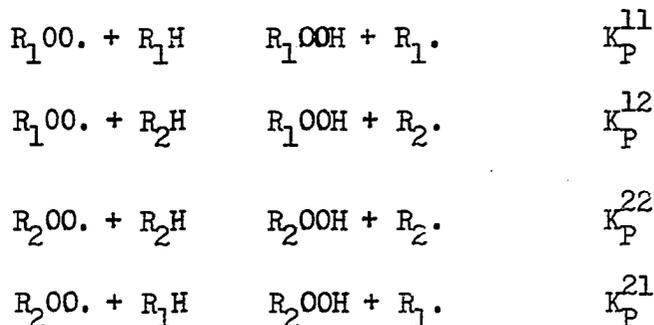
where R_1 represents linoleate, R_2 linolenate, and K_P's are the rate con-
stants. According to Russel (56), the oxidation products at low conversion
allow comparison of rates of the propagation reactions in autoxidation,
essentially free from the effects of chain initiation, termination, and
overall rates. He derived the following equation:

$$\frac{d(R_1H)}{d(R_2H)} = \frac{d(R_1OOH)}{d(R_2OOH)} = \frac{r_1(R_1H/R_2H) + 1}{r_2(R_2H/R_1H) + 1}$$

where the reactivity ratio

$$r_1 = K^{11}/K^{12}$$
$$r_2 = K^{22}/K^{21} .$$

If $(R_1OOH)/(R_2OOH)$ is measured at several values of $(R_1H)/(R_2H)$, and
the parameters ρ and R are defined as:

$$\rho = \frac{(R_1OOH)}{(R_2OOH)}$$

$$R = \frac{(R_1H)}{(R_2H)}$$

than a plot of $(\rho-1)/R$ vs. ρ/R^2 should give a straight line with slope
of $-r_2$ and intercept of r_1 (56). Figure 7 shows the line for the linoleate-
linolenate mixtures. The slope is -2.0639 and intercept of 0.44425.
$r_1 \cdot r_2$ should be about one (56). In this case, it was 0.92.

A similar plot of the oleate-linoleate results fails to give a line
because of the error in analysis at low oleate concentration.

Figure 7. Finemann-Ross plot of linoleate-linolenate cooxidation
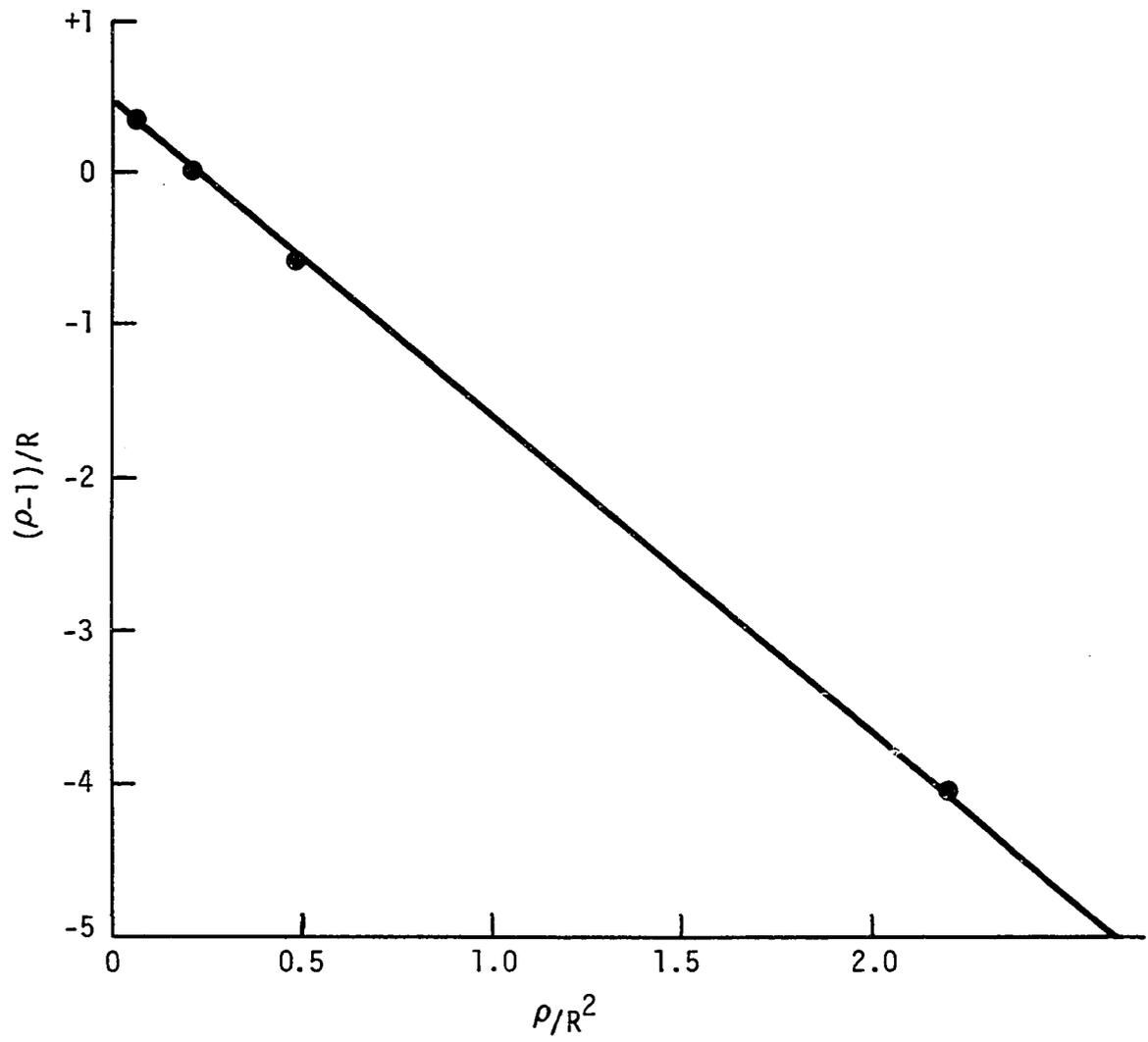
Oxidation of Ternary Mixtures of Fatty Acid Esters

Various mixtures of methyl oleate, linoleate, and linolenate were oxidized, and the peroxides that formed were analyzed quantitatively. The results are in Table 4.

In the mixtures of three fatty acids, the oxidation pattern was similar to that of the binary mixtures. Minor deviations from a constant ratio of oxidation rates could be due to experimental errors. In mixtures containing low concentrations of oleate, relatively more oxidized oleate was found. This could be due to contamination with the impurity mentioned before. The extent of oxidation, up to a peroxide value of about 70, did not have any significant effect on the relative oxidation rate of fatty acid esters.

Because of poor yields of TMS-linolenate, we thought that some decomposition of hydroperoxide might happen at 28 C, the oxidation temperature that was used. Five samples of binary and ternary mixtures of fatty acid esters were oxidized at 21 C. However, the results (Table 5) were not significantly different from those at 28 C.

Surprisingly, these results agree closely with the relative oxygen absorption rates for these fatty acids that were found by Gunston and Hilditch (24) and Stirton et al. (60). They found relative rates of oxygen uptake of oleate:linoleate:linolenate of 1:10-12:25. For some mixtures, our results are quite different from those reported by Frankel et al. (20,21), but these differences could be partly due to the conditions and extent of oxidation. The great discrepancies between the present results and Johnson's data (36) may stem from the separation achieved on silver ion plates. As Figure 1 shows, silver ion plates cannot completely

Table 4. GLC analysis of the formation of peroxide types in autoxidized oleate-linoleate-linolenate mixtures

Composition methyl ester mixture					Peroxide %			Corected peroxide ratio[a]	
18:1	18:2	18:3	P.V.	Recovery %	Monoene	Diene	Triene	18:2/18:1	18:3/18:2
89.37	5.54	5.09	42.3	94.3	34.54	21.82	43.64	10.2	1.83
89.33	7.31	3.35	44.2	102.1	36.62	32.39	30.99	10.8	2.08
89.23	3.75	7.02	41.2	96.7	35.23	14.77	50.00	9.97	1.8
79.48	10.42	10.1	56.8	91.9	23.3	29.13	47.57	9.54	1.7
79.38	13.79	6.83	40.3	94.5	22.98	41.92	35.09	10.5	1.7
			55.3	89.7	21.9	43.81	34.29	11.5	1.6
79.22	7.14	13.64	49.8	87.1	20.2	18.31	61.48	10.05	1.75
			59.9	85.9	18.48	19.18	62.33	11.5	1.7
69.17	15.54	15.29	45.2	90.8	13.92	27.84	58.23	8.9	2.1
			64.5	89.7	11.54	30.12	58.33	11.6	1.96
69.11	20.70	10.19	39.9	86.4	14.35	42.58	43.06	9.9	2.05
			69.7	87.7	12.10	44.37	43.57	12.2	2
69.23	10.63	20.14	38.6	91.5	13.14	17.81	69.05	8.8	2.05
			53.9	87.6	11.91	20.30	67.69	11.1	1.8
5.33	88.9	5.77	52.6	105.2	---	86.03	13.97	---	2.5
13.27	70.84	15.89	36.5	104.4	1.97	67.83	30.19	6.5	1.98
22.60	52.12	25.28	65.9	92.4	2.39	52.56	45.05	9.5	1.8

33.67	30.17	36.16	41.6	88.3	3.09	27.99	68.92	10.1	2.05
44.01	12.32	43.67	40.4	91.4	4.26	12.52	83.22	10.5	1.9
14.91	15.11	69.97	51.7	89.4	1.56	10.16	88.28	6.4	1.9
22.80	25.75	51.45	35.5	93.4	2.41	17.91	79.68	6.6	2.23
33.26	36.12	30.61	35.9	86.2	4.53	30.74	64.72	11.2	1.8
42.44	46.42	11.14	37.9	90.2	6.06	62.88	31.06	9.5	2.06

[a]Calculated as in Table 2.

Table 5. GLC analysis of the formation of peroxide in fatty acid mixtures, oxidized at 21 C

Composition methyl ester mixture					Peroxide %			Corrected peroxide ratio[a]	
18:1	18:2	18:3	P.V.	Recovery %	Monoene	Diene	Triene	18:2/18:1	18:3/18:2
78.53	8.41	13.05	36.7	93.7	21.78	20.79	57.43	8.9	1.8
42.51	46.97	10.52	45.9	90.4	5.30	64.21	30.49	10.9	2.12
5.39	87.86	6.75	41.35	94.7	---	85.29	14.71	---	2.26
77.31	22.69	---	22.5	89.4	24.52	75.48	---	10.4	---
---	49.16	50.84	48.7	104.2	---	30.03	69.97	---	2.27

[a]Calculated as in Table 2.

separate linoleate from oleate. Apparently, Johnson confused one of the

hydroxydiene isomers with monoene in his separation.

Generally, these results support the idea that the ease of ab-

straction of hydrogen from the olefin should be the controlling factor in

autoxidation more than the nature of the attacking alkylperoxy radical (56).

Triglyceride Structure and Oxidation

It has been suggested that triglyceride structure could affect the

susceptibility of fatty acids to oxidation (53, 57). To study this, soy-

bean and olive oils were purified with alumina column. Samples of these

two oils were also randomized with a sodium methoxide catalyst and then

purified with aluminum. In randomization, the fatty acids are detached

from their original position on glycerol and rearranged in a random

manner. This changes the proportions of the fatty acids at the 1, 2, and

3 positions of glycerol. The fatty acids composition of the oils are

given in Table 6.

Table 6. Fatty acid composition of methyl esters of oils by gas
chromatography by per cent

Fatty Acids	Olive Oil	Randomized Olive Oil	Soybean Oil	Randomized Soybean Oil
Saturated	15.68	16.20	13.67	13.61
Oleic	74.02	73.16	25.87	25.44
Linoleic	10.30	10.64	52.98	53.24
Linolenic	--	--	7.47	7.71

The purified oils were oxidized, and after reduction, they were

converted to methyl esters by transesterification in methanol with sodium

methoxide. The results of the autoxidations of the natural and randomized

oils are given in Table 7.

The differences between the relative oxidation rates of the fatty acids in natural and randomized oils were within experimental error. The results for both natural and randomized oils are also very similar to those that were found for the corresponding fatty acid methyl ester mixtures. The results obtained for natural oils are somewhat in contrast to the findings of Raghuveer (53) and Johnson (36). They found that fatty acids in natural oils often oxidized in different proportions than would be predicted from oxidation of the corresponding methyl ester mixtures. The present results show that glyceride structure does not greatly influence the proportions of the peroxides that are formed. But if the effect of glyceride structure is to alter the relative rates of oxidation of the sn-2 versus the sn-1 and -3 positions as Raghuveer (53) supposed, a calculation shows that for these oils the departure of the fatty acids from a nonrandom distribution is probably insufficient to cause a variation in the proportions of peroxide that would exceed experimental error. Randomized oils or methyl ester mixtures oxidized three of four times faster than natural oils. The reason for this is not clear.

Table 7. GLC analysis of the effect of triglyceride structure on the formation of peroxide types

Oil	P.V.	Recovery %	Peroxide %			Corrected peroxide ratio[a]	
			Monoene	Diene	Triene	13:2/18:1	18:3/18:2
Olive oil	31.3	89.1	39.02	60.98	---	11.22	---
Randomized olive oil	40.6	91.6	40.50	59.50	---	10.09	---
Soybean oil	39.5	89.2	4.77	73.55	21.67	7.52	2.09
Randomized soybean oil	36.1	87.4	4.57	72.87	22.54	7.7	2.13

[a]Calculated as in Table 2.

SUMMARY

Pure methyl oleate, linoleate, and linolenate were oxidized in 5 g lots at 28 C. Samples were withdrawn periodically to determine peroxide value by the method of Hamm et al. (27). When the test gave peroxide values of approximately 5, 10, 20, and 40, samples were withdrawn and peroxides were reduced to alcohols with aqueous potassium iodide (48). The hydroxyesters were separated from unoxidized esters by thin-layer chromatography and converted to the TMS-derivatives. The purity and identity of the TMS-linolenate were demonstrated by infrared spectroscopy and mass spectrometry. The TMS-derivatives of pure hydroxyesters were analyzed by gas chromatography using methyl heptadecanoate as an internal standard. Apparent yields for methyl oleate, linoleate, and linolenate were 62%, 44%, and 17% respectively.

A series of binary and ternary mixtures of oleate, linoleate, and linolenate were oxidized to peroxide value of approximately 40, and the peroxides formed were analyzed quantitatively. The results showed that linoleate oxidized about 10.3 times faster than oleate, and linolenate oxidized two times faster than linoleate. In mixtures low in oleate, the oxidation rate of this fatty acid seemed higher. GLC analysis showed the presence of an impurity that could account for this deviation.

The autoxidation of natural oils showed that glyceride structure did not greatly affect the peroxide types. However, natural oils oxidized much slower than randomized oils or methyl esters mixtures.

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